



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/11, 15/63, 15/82</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/32619</b>
			(43) International Publication Date: 1 July 1999 (01.07.99)

<p>(21) International Application Number: PCT/US98/27233</p> <p>(22) International Filing Date: 21 December 1998 (21.12.98)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>60/068,562</td> <td>23 December 1997 (23.12.97)</td> <td>US</td> </tr> <tr> <td>09/215,257</td> <td>18 December 1998 (18.12.98)</td> <td>US</td> </tr> </table> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</p> <table border="0"> <tr> <td>US</td> <td>60/068,562 (CON)</td> </tr> <tr> <td>Filed on</td> <td>23 December 1997 (23.12.97)</td> </tr> <tr> <td>US</td> <td>09/215,257 (CON)</td> </tr> <tr> <td>Filed on</td> <td>18 December 1998 (18.12.98)</td> </tr> </table> <p>(71) Applicants (for all designated States except US): THE CARNEGIE INSTITUTE OF WASHINGTON [US/US]; 1530 P Street, N.W., Washington, DC 20005 (US). THE UNIVERSITY OF MASSACHUSETTS [US/US]; One Beacon Street, Boston, MA 02108 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): FIRE, Andrew [US/US]; 2320 Bright Leaf Way, Baltimore, MD 21210 (US). XU, Siqun [CN/US]; 1755 Warminton Court, Ballwin, MO</p>	60/068,562	23 December 1997 (23.12.97)	US	09/215,257	18 December 1998 (18.12.98)	US	US	60/068,562 (CON)	Filed on	23 December 1997 (23.12.97)	US	09/215,257 (CON)	Filed on	18 December 1998 (18.12.98)	<p>63021 (US). MONTGOMERY, Mary, K. [US/US]; 233 Macalester Street, St. Paul, MN 55105 (US). KOSTAS, Stephen, A. [US/US]; 126 East Melrose Avenue, Baltimore, MD 21212 (US). TIMMONS, Lisa [US/US]; 2408 Brambleton Road, Baltimore, MD 21209 (US). TABARA, Hiroaki [JP/US]; Apartment #1, 145 Orient Street, Worcester, MA 01604 (US). DRIVER, Samuel, E. [US/US]; Apartment #4, 1714 Commonwealth Avenue, Brighton, MA 02135 (US). MELLO, Craig, C. [US/US]; 19 Ryan Road, Shrewsbury, MA 01545 (US).</p> <p>(74) Agents: KOKULIS, Paul, N. et al.; Pillsbury Madison &amp; Sutro LLP, 1100 New York Avenue, N.W., Washington, DC 20005 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b></p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
60/068,562	23 December 1997 (23.12.97)	US													
09/215,257	18 December 1998 (18.12.98)	US													
US	60/068,562 (CON)														
Filed on	23 December 1997 (23.12.97)														
US	09/215,257 (CON)														
Filed on	18 December 1998 (18.12.98)														

(54) Title: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

(57) Abstract

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced *ex vivo* or *in vivo*. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## GENETIC INHIBITION BY DOUBLE-STRANDED RNA

## GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

## 2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to

produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

#### Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stoichiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

### Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

### Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been

hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering

5 RNA, as well as the nature of the interference process, have not been determined.

Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous

10 gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

#### Distinction between the Present Invention and Antisense Approaches

15 The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in

20 the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by

25 double-stranded RNA must occur by a mechanism distinct from antisense interference.

#### Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur

30 rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated

inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition in *C. elegans* is mediated by a triple-strand structure.

#### Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

## SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a

5 nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a  
10 transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA  
15 may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The  
20 double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding  
25 to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference  
30 between the nucleotide sequences. Alternatively, the duplex region of the RNA may be



defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease

models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22*<sup>9</sup>, *unc-54*<sup>12</sup>, *fem-1*<sup>14</sup>, and *hlh-1*<sup>15</sup>).

Figures 2 A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (*ds-unc22A*). Panels D-F show progeny of animals injected with *ds-gfpG*. Panels G-I demonstrate specificity. Animals are injected with *ds-lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20  $\mu$ m.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA<sup>20</sup>). Panel C: Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA was detected. Scale: each embryo is approximately 50  $\mu$ m in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0-6 hours, 2 for 6-15

hours, 3 for 15-27 hours, 4 for 27-41 hours, and 5 for 41-56 hours. The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids<sup>8,3</sup>.

Figures 5 A-C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)<sup>28</sup> expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The

consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme  
5 linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP),  
10 beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphino-  
15 thracin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition  
20 in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or trans-  
25 lated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For  
30 example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored

to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or  
5 organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or  
10 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene  
15 are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference  
20 between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a  
25 nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene  
30 is not required to practice the present invention. Thus the invention has the advantage of

being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may  
5 be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton,  
10 flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, black-  
berry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit,  
15 guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

20 Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nema-  
25 todirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphelenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynychus, Tylenchus, and Xiphinema). Representative  
30 orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art<sup>32, 33, 34</sup> (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, intro-

duced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the

5 RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression.

10 Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

15 Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA

20 encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the

25 annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene

30 may be selected which is required for initiation or maintenance of the disease/pathology.



Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

5 A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) 10 infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated 15 with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in 20 situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhus, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, 25 Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, 30 dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adeno-

carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyo-  
 5 sarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangio-  
 10 myoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyo- sarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital,  
 15 head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyper- thermia, radiation therapy, and the like.

20 As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation  
 25 factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53,  
 30 and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases,

amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of Plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); *Distribution of Plant-Parasitic Nematode Species in North America* (Society of Nematologists, 1985); and *Fungi on Plants and Plant Products in the United States* (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or

prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vivo* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial  
5 explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes.

10 Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the  
15 target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane  
20 domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

25 The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

30 Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The

pesticide of the present invention may serve as an arachnicide, insecticide, nematocide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath  
5 ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the  
10 objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either a pesticide or nutrient, a formulation of the present invention may  
15 be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension  
20 may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately  
25 with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may  
30 also promote the persistence and/or spread of the formulation.

**Description of the dsRNA Inhibition Phenomenon in *C. elegans***

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to  
5 interfere with function of an endogenous gene<sup>1,2</sup>. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a  
10 variety of studies to manipulate gene expression<sup>3,4</sup>.

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the litera-  
15 ture was that sense and antisense RNA preparations are each sufficient to cause interference<sup>3,4</sup>. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic  
20 inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference<sup>3,4</sup> had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target  
25 gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for *unc-22* and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had  
30 only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro*



promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To  
5 rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention;  
10 however, the practice of the invention is not limited or restricted in any way by them.

#### **Analysis of RNA-Mediated Inhibition of *C. elegans* Genes**

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene  
15 activity and the movement phenotypes of animals<sup>3,8</sup>: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein<sup>7-9</sup>. *unc-22* mRNA is present at several thousand copies per striated muscle cell<sup>3</sup>.

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only  
20 marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (Figure 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The  
25 lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect forma-  
30 tion of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predomi-

nantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism<sup>10</sup>. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction<sup>7,11,12</sup>, *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production<sup>13,14</sup>, and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility<sup>15,16</sup>. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and

larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes<sup>17</sup>. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells<sup>11,16</sup>. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles<sup>18,19</sup>). At high concentrations of *gfp* dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or post-embryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, a *mex-3* transcript that is abundant in the gonad and early embryos<sup>20</sup> was targeted, where straightforward *in situ* hybridization can be performed<sup>5</sup>. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3* (Figure 3D), but injection of purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see Figure 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals

with dsRNA.

The *C. elegans* gene *unc-22* encodes an abundant muscle filament protein. *unc-22* null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from *unc-22*, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the *unc-22* gene. The *C. elegans* *fem-1* gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to *fem-1*, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a *gfp* transgene were fed bacteria expressing dsRNA corresponding to the *gfp* reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see Figure 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from *fem-1* and *gfp* produced no twitching, dsRNAs from *unc-22* and *fem-1* did not reduce *gfp* expression, and dsRNAs from *gfp* and *unc-22* did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either *gfp* or *unc-22* caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The *unc-22* dsRNA was segment ds-*unc22A* from Figure 1. *pos-1* and *sqt-3* dsRNAs were from the full length cDNA clones. *pos-1* encodes an essential maternally provided component required early in embryogenesis. Mutations removing *pos-1* activity have an early embryonic arrest characteristic of *skn*-like mutations<sup>29, 30</sup>. Cloning and activity patterns for *sqt-3* have been described<sup>31</sup>. *C. elegans* *sqt-3* mutants have mutations in the *col-1* collagen gene<sup>31</sup>. Phenotypes of affected animals are noted. Incidences of

clear phenotypic effects in these experiments were 5-10% for *unc-22*, 50% for *pos-1*, and 5% for *sqt-3*. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that *unc-22* dsRNA produced only Unc-22 phenotypes, *pos-1* dsRNA produced only Pos-1 phenotypes, and *sqt-3* dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. *Nature*, 391, 806-811, 1998; Timmons, L. & Fire, A. *Nature*, 395, 854, 1998; and Montgomery, M.K. & Fire, A. *Trends in Genetics*, 14, 255-258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions<sup>21</sup> for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

#### Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase<sup>6</sup>, followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68°C to remove secondary structure, sense+antisense annealing was carried out in injection buffer<sup>27</sup> at 37°C for 10-30 minutes.

Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory effect and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of  $0.5 \times 10^6$  to  $1.0 \times 10^6$  molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

### Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp*<sup>27</sup> and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone<sup>26</sup> as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between

cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncytium of adults and expelling 20-100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

### Additional Description of the Results

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: *unc-22*<sup>9</sup>, *unc-54*<sup>12</sup>, *fem-1*<sup>14</sup>, and *hlh-1*<sup>15</sup>). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (Figure 2, panels A-H). These experiments were carried out in a reporter strain (called PD4251) expressing



two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. *ds-unc22A* RNA was injected as a negative control.

5 GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (Figure 2A), adult (Figure 2B), and adult body wall at high magnification (Figure 2C).

10 In contrast, the progeny of animals injected with *ds-gfpG* RNA are affected (Figures 2D-F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (Figure 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult  
15 animal (Figure 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in Figure 2F). Inhibition was target specific (Figures 2G-I). Animals were injected with *ds-lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ  
20 was absent from almost all cells (larva in Figure 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (Figure 2H). Scale bars in Figure 2 are 20  $\mu$ m.

The effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA was shown by *in situ* hybridization to embryos (Figure 3, panels A-D).  
25 D). The 1262 nt *mex-3* cDNA clone<sup>20</sup> was divided into two segments, *mex-3A* and *mex-3B* with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3B* antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and *in situ* hybridization (see Reference 5). The  
30 *mex-3B* dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3A* were used to

assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (Figure 3A). Embryos from uninjected parents showed a normal pattern of endogenous *mex-3* RNA (Figure 3B). The observed pattern of *mex-3* RNA was as previously described in Reference 20. Injection of purified *mex-3*B antisense RNA produced at most a modest effect: the resulting embryos retained *mex-3* mRNA, although levels may have been somewhat less than wild type (Figure 3C). In contrast, no *mex-3* RNA was detected in embryos from parents injected with dsRNA corresponding to *mex-3*B (Figure 3D). The scale of Figure 3 is such that each embryo is approximately 50  $\mu$ m in length.

Gene-specific inhibitory activity by *unc-22A* RNA was measured as a function of RNA structure and concentration (Figure 4). Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids<sup>8,3</sup>.

25

Figures 5 A-C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (Figure 5A). A bacterial strain (BL21/DE3)<sup>28</sup> expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected

30

bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (Figure 5B). PD4251 animals were also reared on a diet of bacteria expressing  
 5 dsRNA corresponding to the coding region for *gfp*. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (Figure 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either *unc-22* or *gfp*. This was comparably effective.

10

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

- 15 1. Izant, J. & Weintraub, H. *Cell* 36, 1007-1015 (1984).
2. Nellen, W. & Lichtenstein, C. *TIBS* 18, 419-423 (1993).
3. Fire, A., et al. *Development* 113, 503-514 (1991).
4. Guo, S. & Kemphues, K. *Cell* 81, 611-620 (1995).
5. Seydoux, G. & Fire, A. *Development* 120, 2823-2834 (1994).
- 20 6. Ausubel, F., et al. *Current Protocols in Molecular Biology*, John Wiley N.Y. (1990).
7. Brenner, S. *Genetics* 77, 71-94 (1974).
8. Moerman, D. & Baillie, D. *Genetics* 91, 95-104 (1979).
9. Benian, G., et al. *Genetics* 134, 1097-1104 (1993).
10. Proud, C. *TIBS* 20, 241-246 (1995).
- 25 11. Epstein H., et al. *J. Mol. Biol.* 90 291-300 (1974).
12. Karn, J., et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 80, 4253-4257 (1983).
13. Doniach, T. & Hodgkin J.A. *Dev. Biol.* 106, 223-235 (1984).
14. Spence, A., et al. *Cell* 60, 981-990 (1990).
15. Krause, M., et al. *Cell* 63, 907-919 (1990).
- 30 16. Chen, L., et al. *Development*, 120, 1631-1641 (1994).
17. Dibb, N. J., et al. *J. Mol. Biol.* 205, 603-613 (1989).

18. Sulston, J., et al. *Dev. Biol.* 100, 64-119 (1983).
19. Sulston, J. & Horvitz, H. *Dev. Biol.* 82, 41-55 (1977).
20. Draper B.W., et al. *Cell* 87, 205-216 (1996).
21. Sulston, J., et al. *Nature* 356, 37-41 (1992).
- 5 22. Matzke, M. & Matzke, A. *Plant Physiol.* 107, 679-685 (1995).
23. Ratcliff, F., et al. *Science* 276, 1558-1560 (1997).
24. Latham, K. *Trends in Genetics* 12,134-138 (1996).
25. Mello, C. & Fire, A. *Methods in Cell Biology* 48, 451-482 (1995).
26. Clark, D., et al. *Mol. Gen. Genet.* 247, 367-378 (1995).
- 10 27. Chalfie, M., et al. *Science* 263, 802-805 (1994).
28. Studier, F., et al. *Methods in Enzymology* 185, 60-89 (1990).
29. Bowerman, B., et al. *Cell* 68, 1061-1075 (1992).
30. Mello, C.C., et al. *Cell* 70, 163-176 (1992).
31. van der Keyl, H., et al. *Develop. Dynamics* 201, 86-94 (1994).
- 15 32. Goeddel, D.V. *Gene Expression Technology*, Academic Press, 1990.
33. Kriegler, M. *Gene Transfer and Expression*, Stockton Press, 1990.
34. Murray, E.J. *Gene Transfer and Expression Protocols*, Humana Press, 1991.

Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

	Gene and Segment		Size	Injected RNA	F1 Phenotype
5	<i>unc-22</i>			<i>unc-22</i> null mutants: strong twitchers <sup>7,8</sup>	
	<i>unc22A</i> <sup>a</sup>	exon 21-22	742	sense	wild type
				antisense	wild type
				sense+antisense	strong twitchers (100%)
	<i>unc22B</i>	exon 27	1033	sense	wild type
10				antisense	wild type
				sense+antisense	strong twitchers (100%)
	<i>unc22C</i>	exon 21-22 <sup>b</sup>	785	sense+antisense	strong twitchers (100%)
	<i>fem-1</i>			<i>fem-1</i> null mutants: female (no sperm) <sup>13</sup>	
15	<i>fem1A</i>	exon 10 <sup>c</sup>	531	sense	hermaphrodite (98%)
				antisense	hermaphrodite (>98%)
				sense+antisense	female (72%)
	<i>fem1B</i>	intron 8	556	sense+antisense	hermaphrodite (>98%)
20	<i>unc-54</i>			<i>unc-54</i> null mutants: paralyzed <sup>7,11</sup>	
	<i>unc54A</i>	exon 6	576	sense	wild type (100%)
				antisense	wild type (100%)
				sense+antisense	paralyzed (100%) <sup>d</sup>
	<i>unc54B</i>	exon 6	651	sense	wild type (100%)
25				antisense	wild type (100%)
				sense+antisense	paralyzed (100%) <sup>d</sup>
	<i>unc54C</i>	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
	<i>unc54D</i>	promoter	567	sense+antisense	wild type (100%)
	<i>unc54E</i>	intron 1	369	sense+antisense	wild type (100%)
30	<i>unc54F</i>	intron 3	386	sense+antisense	wild type (100%)

Table 1 (continued).

	Gene and Segment		Size	Injected RNA	F1 Phenotype
5	<b><i>hlh-1</i></b>			<b><i>hlh-1</i> null mutants: lumpy-dumpy larvae<sup>16</sup></b>	
	<i>hlh1A</i>	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
				antisense	wild type (<2% lpy-dpy)
				sense+antisense	lpy-dpy larvae (>90%) <sup>e</sup>
	<i>hlh1B</i>	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%) <sup>e</sup>
10	<i>hlh1C</i>	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%) <sup>e</sup>
	<i>hlh1D</i>	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
<hr/>					
	<b><i>myo-3</i> driven GFP transgenes<sup>f</sup></b>				
	<b><i>myo-3::NLS::gfp::lacZ</i></b>			<b>makes nuclear GFP in body muscle</b>	
15	<i>gfpG</i>	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
				antisense	nuclear GFP-LacZ pattern of parent strain
				sense+antisense	nuclear GFP-LacZ absent in 98% of cells
	<i>lacZL</i>	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
20	<b><i>myo-3::MtLS::gfp</i></b>			<b>makes mitochondrial GFP in body muscle</b>	
	<i>gfpG</i>	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
				antisense	mitochondrial GFP pattern of parent strain
				sense+antisense	mitochondrial GFP absent in 98% of cells
	<i>lacZL</i>	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

25

## Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites ( $0.5-1 \times 10^6$  molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were

scored upon hatching and subsequently at 12-24 hour intervals.

30

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested ( $3.6 \times 10^6$  molecules per gonad), the

individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

5           b: *unc22C* also carries the intervening intron (43 nt).

          c: *fem1A* also carries a portion (131 nt) of intron 10.

          d: Animals in the first affected broods (laid at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null  
10       mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

          e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially  
15       elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

          f: The host for these injections, PD4251, expresses both mitochondrial GFP and  
20       nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
<i>unc22B</i>	Gonad	weak twitchers	strong twitchers
<i>unc22B</i>	Body Cavity Head	weak twitchers	strong twitchers
<i>unc22B</i>	Body Cavity Tail	weak twitchers	strong twitchers
<i>g/pG</i>	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
<i>g/pG</i>	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
<i>lacZL</i>	Gonad	lower nuclear GFP	rare or absent nuclear GFP
<i>lacZL</i>	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP



Table 3. *C. elegans* can respond in a gene-specific manner to environmental dsRNA.

5	<b>Bacterial Food</b>	<b>Movement</b>	<b>Germline Phenotype</b>	<b>GFP-Transgene Expression</b>
	BL21(DE3)	0% twitch	< 1% female	< 1% faint GFP
	BL21(DE3) [ <i>fem-1</i> dsRNA]	0% twitch	43% female	< 1% faint GFP
	BL21(DE3) [ <i>unc22</i> dsRNA]	85% twitch	< 1% female	< 1% faint GFP
10	BL21(DE3) [ <i>gfp</i> dsRNA]	0% twitch	< 1% female	12% faint GFP

Table 4. Effects of bathing *C. elegans* in a solution containing dsRNA.

15

	<b>dsRNA</b>	<b>Biological Effect</b>
	<i>unc-22</i>	Twitching (similar to partial loss of <i>unc-22</i> function)
20	<i>pos-1</i>	Embryonic arrest (similar to loss of <i>pos-1</i> function)
	<i>sqt-3</i>	Shortened body (Dpy) (similar to partial loss of <i>sqt-3</i> function)

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After *ds-unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with *ds-unc22A*. Injections of *ds-gfpG* or *ds-lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of *ds-gfpG* and *ds-lacZL* produced no twitching, while injections of *ds-unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

## WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA comprises a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.
2. The method of claim 1 in which the target gene is a cellular gene.
3. The method of claim 1 in which the target gene is an endogenous gene.
4. The method of claim 1 in which the target gene is a transgene.
5. The method of claim 1 in which the target gene is a viral gene.
6. The method of claim 1 in which the cell is from an animal.
7. The method of claim 1 in which the cell is from a plant.
8. The method of claim 6 in which the cell is from an invertebrate animal.
9. The method of claim 8 in which the cell is from a nematode.
10. The method of claim 1 in which the identical nucleotide sequence is at least 50 bases in length.
11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.
12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of claim 1 in which the RNA comprises one strand which is self-complementary.
14. The method of claim 1 in which the RNA comprises two separate complementary strands.
15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.
16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.
17. The method of claim 1 in which the cell is present in an organism, and the RNA is introduced within a body cavity of the organism and outside the cell.
18. The method of claim 1 in which the cell is present in an organism and the RNA is introduced by extracellular injection into the organism.
19. The method of claim 1 in which the cell is present in a first organism, and the RNA is introduced to the first organism by feeding a second, RNA-containing organism to the first organism.
20. The method of claim 19 in which the second organism is engineered to produce an RNA duplex.
21. The method of claim 1 in which an expression construct in the cell produces the RNA.
22. A method to inhibit expression of a target gene comprising:
  - (a) providing an organism containing a target cell, wherein the target cell contains the target gene and the target gene is expressed in the target cell;

- (b) contacting a ribonucleic acid (RNA) with the organism, wherein the RNA is comprised of a double-stranded structure with duplexed ribonucleic acid strands and one of the strands is able to duplex with a portion of the target gene; and
  - (c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.
23. The method of claim 22 in which the organism is an animal.
24. The method of claim 22 in which the organism is a plant.
25. The method of claim 22 in which the organism is an invertebrate animal.
26. The method of claim 22 in which the organism is a nematode.
27. The method of claim 26 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.
28. The method of claim 22 in which the identical nucleotide sequence is at least 50 nucleotides in length.
29. The method of claim 22 in which the expression of the target gene is inhibited by at least 10%.
30. The method of claim 22 in which the RNA is introduced within a body cavity of the organism and outside the target cell.
31. The method of claim 22 in which the RNA is introduced by extracellular injection into the organism.

32. The method of claim 22 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

33. The method of claim 32 in which a genetically-engineered host transcribing the RNA comprises the food.

34. The method of claim 22 in which at least one strand of the RNA is produced by transcription of an expression construct.

35. The method of claim 35 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

36. A cell containing an expression construct,  
wherein the expression construct transcribes at least one ribonucleic acid (RNA) and the RNA forms a double-stranded structure with duplexed strands of ribonucleic acid, whereby said cell contains the double-stranded RNA structure and is able to inhibit expression of a target gene when the RNA is contacted with an organism containing the target gene.

37. A transgenic animal containing said cell of claim 36.

38. A transgenic plant containing said cell of claim 36.

39. A kit comprising reagents for inhibiting expression of a target gene in a cell,  
wherein said kit comprises a means for introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, and  
wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

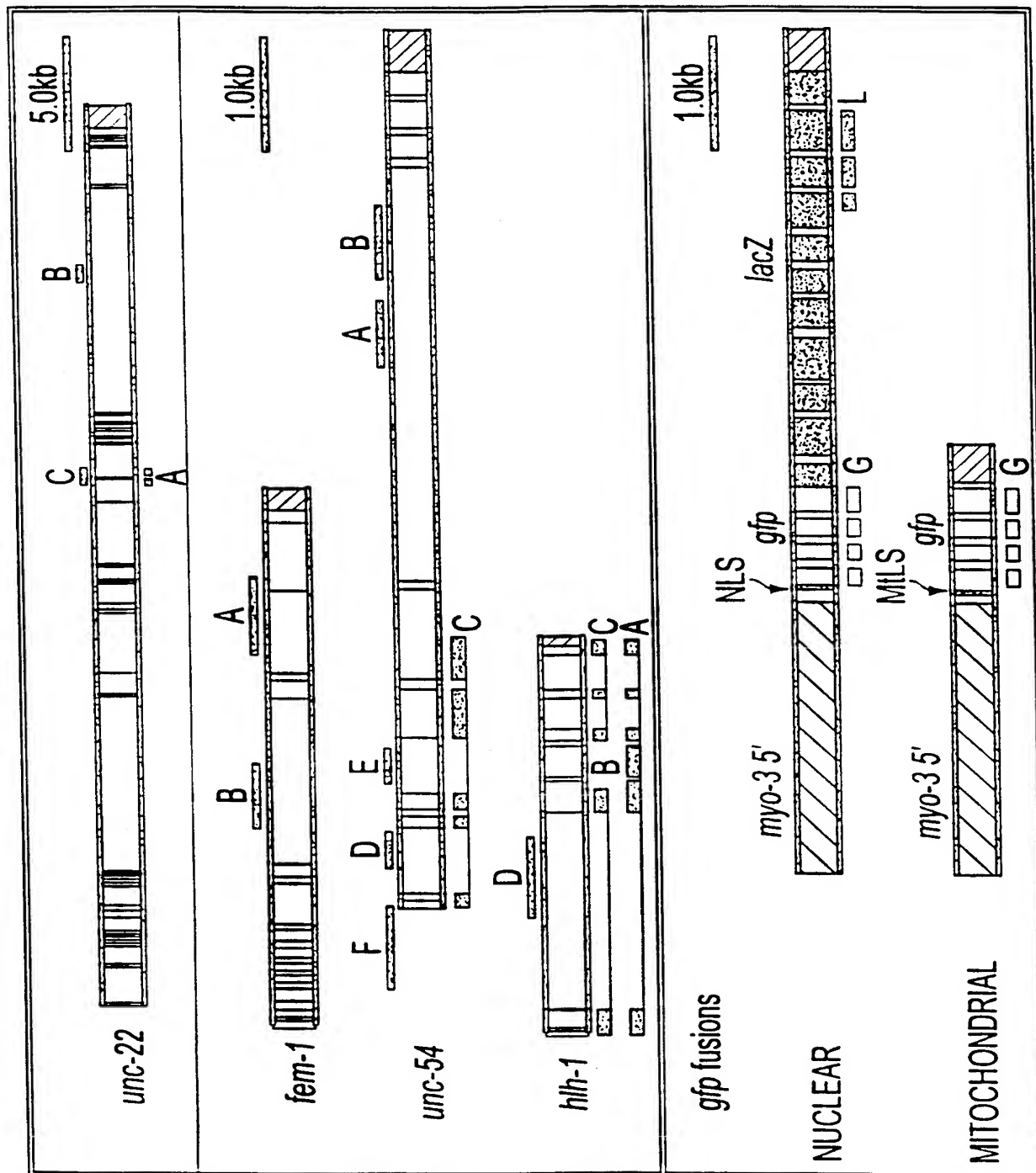


FIG. 1



FIG. 2A

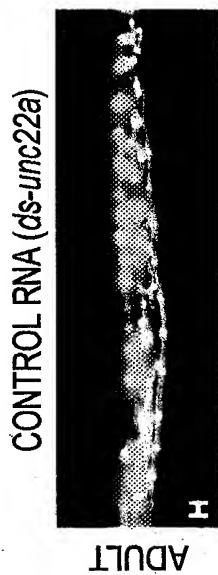


FIG. 2B

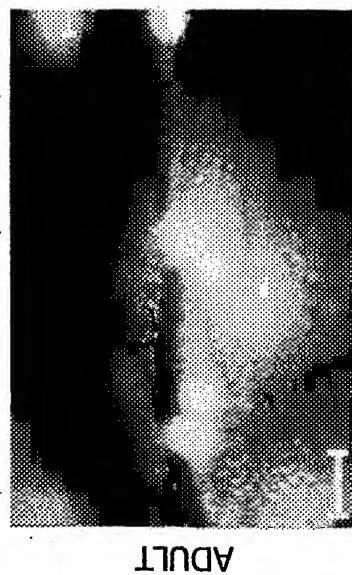


FIG. 2C



FIG. 2D



FIG. 2E

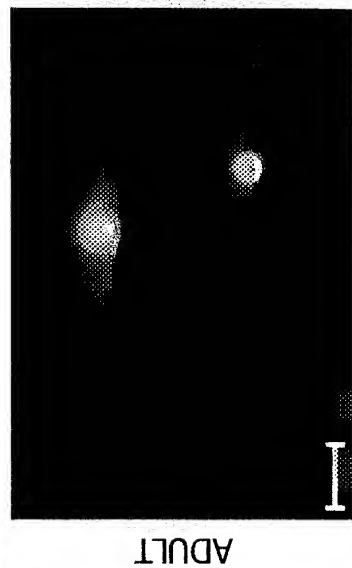


FIG. 2F



FIG. 2G



FIG. 2H

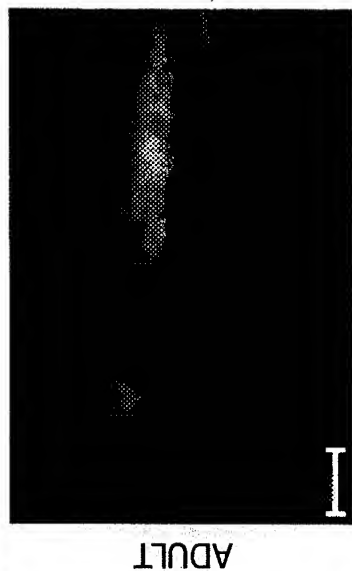


FIG. 2I



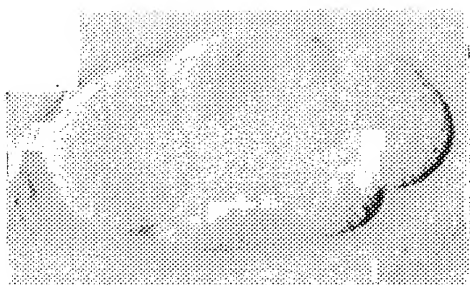


FIG. 3A



FIG. 3B

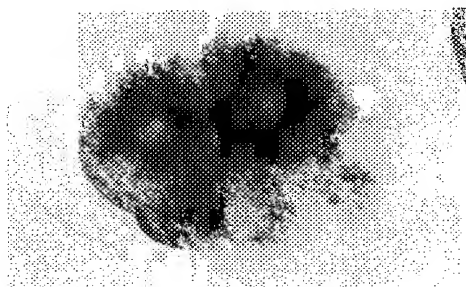
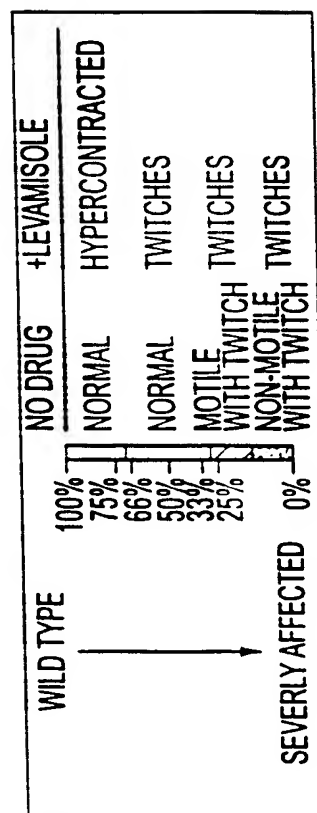
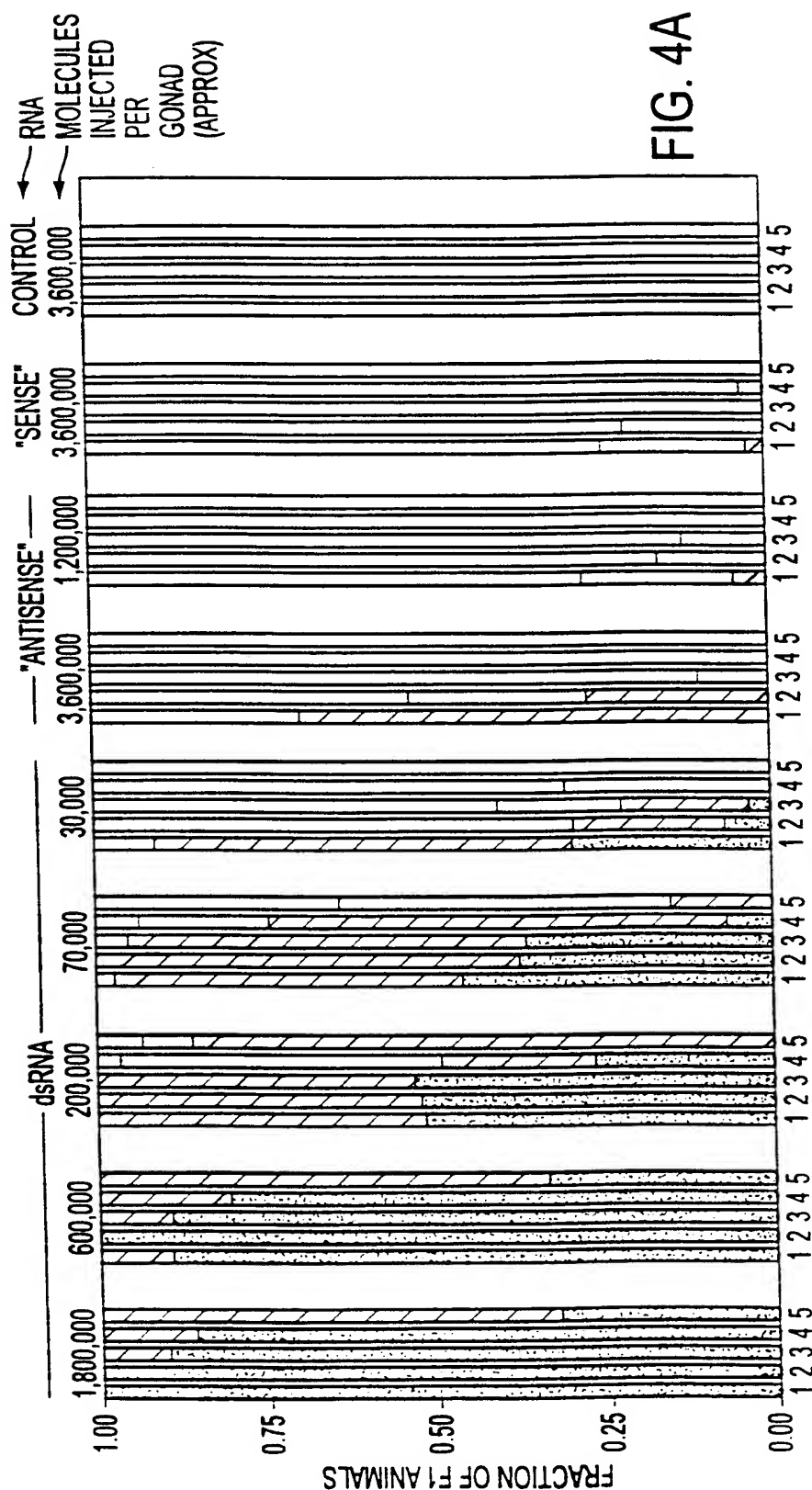


FIG. 3C



FIG. 3D

4/5



PD4251 WORMS FED BACTERIA  
EXPRESSING *gfp* dsRNA

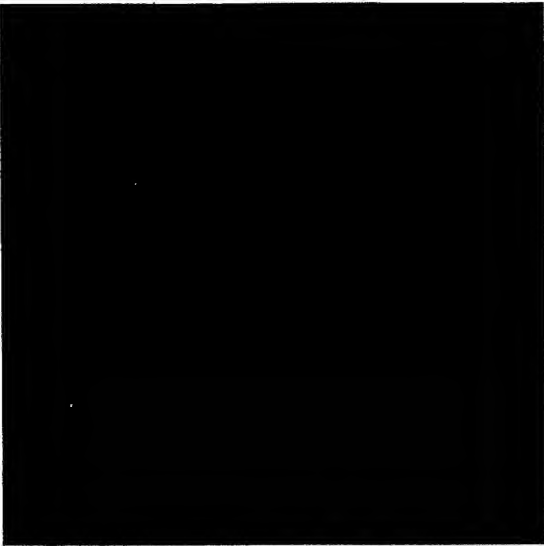


FIG. 5C

PD4251 WORMS

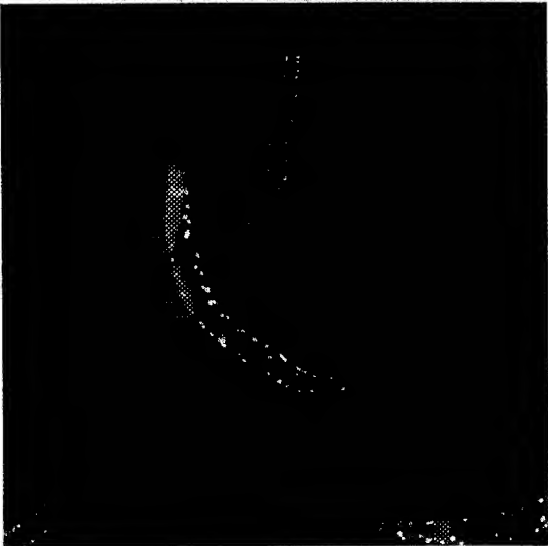


FIG. 5B

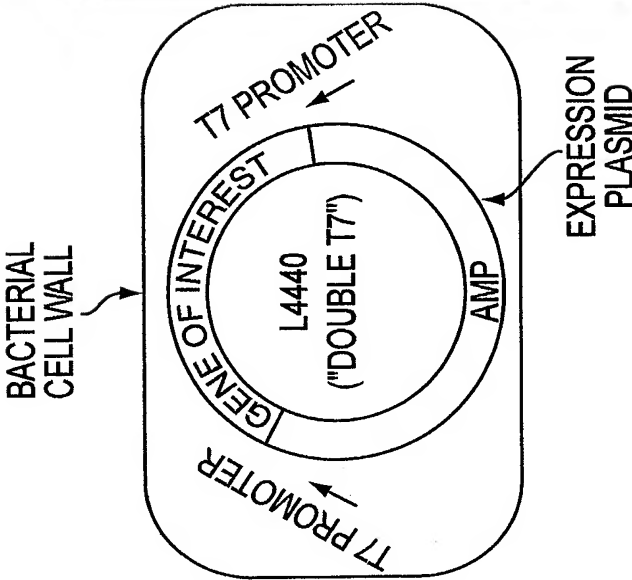


FIG. 5A

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/27233

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N15/63 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RATCLIFF F ET AL: "A similarity between viral defense and gene silencing in plants"</p> <p>SCIENCE,</p> <p>vol. 276, no. 93, 6 June 1997, pages 1558-1560, XP002095874</p> <p>see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

25 May 1999

Date of mailing of the international search report

10/06/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

ANDRES, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/27233

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FIRE, A. ET AL.: "Production of antisense RNA leads to effective and specific inhibition of gene expression in <i>C. elegans</i> muscle"</p> <p>DEVELOPMENT (CAMBRIDGE, UK) (1991), 113(2), 503-14, XP002103600</p> <p>cited in the application</p> <p>see page 508, right-hand column, paragraph 2</p> <p>see page 509, right-hand column - page 511, right-hand column</p> <p>see page 512, 'Discussion' and figure 7</p> <p style="text-align: center;">---</p>	1-39
A	<p>MATZKE M A ET AL: "HOW AND WHY DO PLANTS INACTIVATE HOMOLOGOUS (TRANS)GENES?"</p> <p>PLANT PHYSIOLOGY, vol. 107, no. 3, 1 March 1995, pages 679-685, XP002021174</p> <p>see page 680, left-hand column, paragraph 3 - right-hand column, paragraph 1</p> <p>see page 682</p> <p style="text-align: center;">---</p>	1
P,X	<p>FIRE A ET AL: "Potent and specific genetic interference by double - stranded RNA in <i>Caenorhabditis elegans</i>"</p> <p>NATURE, (1998 FEB 19) 391 (6669) 806-11., XP002095876</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1-3,6, 8-12, 14-18, 21-23, 25,26, 28-31, 34,39
P,X	<p>MONTGOMERY M K ET AL: "Double - stranded RNA as a mediator in sequence-specific genetic silencing and co - suppression"</p> <p>TRENDS IN GENETICS, (1998 JUL) 14 (7) 255-8., XP004124680</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1-4, 6-12, 14-18, 36-39
P,X	<p>TIMMONS L ET AL: "Specific interference by ingested dsRNA"</p> <p>NATURE, (1998 OCT 29) 395 (6705) 854., XP002103601</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">-----</p>	1-3,6, 8-12, 14-23, 25,26, 28-34, 36,39

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 27233

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 35 and claims 1-6,8-23,25-26,34 (as far as in vivo methods practised on animals are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.